 doi:10.20944/preprints202210.0097.v1

Review

A Proteomics Approach in Farm Animals' Milk: A Comprehensive Analysis

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Abstract: Milk is newborn's food and an emulsion full of all necessary components for neonatal growth. Its consumption is worldwide and is the base for all the dairy products. Because of the latter many new technologies are growing, among them proteomics; in order to give new insights in milk's compounds and to maximize the beneficial potential for consumers' health. In this review, we aimed to gather data of proteomics studies for the majority of dairy animals and elucidate the role of milk bioactive compounds. Furthermore, special reference was made to milk fat globule membrane (MFGM) peptides and the result of thermal treatment in milk proteins. Finally, the proteomic approach regarding adulterations was included in the review.

Keywords: cow milk; sheep milk; goat milk; proteomics methods; bioactive peptides; antimicrobial peptides; allergenic potential; adulterations

Introduction

Milk is known to be one of the most complex and rich in compounds food provided by nature. Milk of all mammals is an emulsion that contains water, proteins, fats, carbohydrates, vitamins and minerals since it is the food for the neonatal, and it shows great variance in its consistency among species, especially among ruminants and non-ruminants ones [1]. The mammary gland's product can be affected from various factors altering its composition and quality. In cases such as different species or phases during lactating period affect the composition of milk and the differences are notably. On the other hand, factors as nutrition, genetic polymorphism and environmental conditions; predispose the milk quality and quantity [1].

Milk is one of the most consumed food products globally with bovine being the dominant type of milk produced. The latter fact don't exclude the production and consumption in smaller amounts of milk originated from animals such as sheep, goats, buffalo and equine species. Specifically, bovine milk represents the 85% of the global milk production followed by buffalo milk with 11%, caprine 2.3%, ovine 1.4% and camel milk 0.2%. Milk coming from other species such as donkey, horses and yaks; is equivalent to less than 0.1% of the worldwide production [1].

Recently, in countries of central and north Europe (Belgium, France, the Netherlands and Norway) milk coming from non-traditional species seems to have increased consumption and use in other industries (e.g. cosmetics and pharmaceutical) [2,3]. In these kinds of milk many therapeutic properties, regarding gastrointestinal or cardiovascular conditions; are attributed based on the molecules they contain. [1]. According to ADA-American dietetic association (USA) and FOSHU - Foods for Specified Health Uses (Japan) describe functional food as "functional foods are recognized to provide additional health

 doi:10.20944/preprints202210.0097.v1

benefits that may reduce\ disease risks and/or promote optimal health" and "foods consumed as part of an ordinary diet, containing functional ingredients and exerting health or physiological effect", respectively [4]. In order to affirm those properties, it is necessary to establish that the responsible active components are present in specific concentration in every commercially available product. However, for milk originated from equine or other species it is difficult to standard the products since are produced in small scale and in local farms and are affected by various factors such as breed, nutrition, weather conditions e.tc. Recently, the idea of raw milk's consumption as beneficial food for health has gained in popularity. The basic argument is that milk is not thermally treated so its components remain intact decreasing the possibility to allergies and increasing the flavor and nutritional quality. On the other hand, foodborne pathogens remain in the raw milk, as thermal treatment is the only way to eliminate them, putting in risk consumer's health. Many studies have reported food poisoning by bacteria such as Campylobacter, Salmonella spp. and Escherichia coli following raw milk consumption[5-7].

The ruminants' milk is rich in caseins whereas the non-ruminants' contains higher proportion of whey proteins as it is indicated by the ratio caseins/whey proteins; making this the distinct characteristic between ruminants and non-ruminants. The casein fraction represents around 80% of bovine and sheep milk proteins, roughly 50% of horse milk proteins and less than 50% of human milk proteins [8-10]. The relative proportion of the main milk casein components differ, not only between ruminants and non-ruminants, but also between ruminant species. Given these different relative proportions, casein micelle characteristics differ as well, in size but also in hydration and mineralization [9]. In different species the milk proteins detected can differ in molecular form and sequence of amino acids (e.g. β-lg has a different molecular form in horse milk compared to ruminants [11]), affecting the digestibility of milk in general, the quality, the stability of proteins under thermal treatment as well as the nutritional value of milk as it is based on the ratio of necessary amino acids in milk proteins [12].

Milk is produced right after parturition in the mammary gland and its main service is to supply the newborn with all the essential elements for growth and development. In addition it provides immunoglobulins and other molecules required for immune response against pathogens. The composition of milk proteins differs between species and breeds, and also lactation stage, to fulfill the needs for development of the growing mammal [8].

In the general definition of proteomics [13,14], milk proteomics involves the analysis of milk protein expression, structure and modifications including genetic variants, changes (naturally occurring and via processing and storage) in levels of phosphorylation, glycosylation and other post-translational modifications (PTMs), and the determination of PTM sites on milk proteins [15,16]. Proteomic methods can be applied to identify bioactive components in whey fraction or trapped in casein micelles, as well as in milk fat globule membrane (MFGM), assess the protein thermotolerance of heated milk and their denaturation or not; and identify adulterations. All these aspects will be discussed in this review.

Proteomic techniques applied to milk

Liquid chromatography (LC) and capillary-electrophoresis (CE) are used to separate proteins from complexed samples and provide better sensitivity and robust results compared to 2DE[17]. LC combined with mass spectrometry (MS) can lead to identification of hydrophobic proteins or others with low molecular weight which may not be detected with 2DE[18]. The use of liquid chromatography (LC) can provide many separation aspects including size exclusion, reversed-phase (RP) and ion exchange for the identification of any protein with unique characteristics [17] after digestion of proteins with proteases (e.g. trypsin, chymosin) for peptide generation. Then, the peptide mixture is analyzed by LC-MS using a C18 reversed phase, capillary column and an electrospray ionization (ESI) source, known as one-dimensional (1-D) LC-MS. B-lactoglobulin's lactosylation sites was

 doi:10.20944/preprints202210.0097.v1

the first milk protein characterized by this technique [19]. By combining different separation modes such as two-dimensional (2-D) (strong cation exchange/reversed phase) or three-dimensional (strong cation exchange/avidin/reversed phase) chromatography, may result in detection of more low abundance proteins and overcome the low resolution of 1-D LC methods [20]. Resolution of highly complex samples such as milk was performed by the application of 2-D LC including in the first dimension a strong cation exchanger to separate peptides followed by reversed phase in the second dimension [21]. That approach was firstly applied for fractioning of Bos taurus' milk proteins using a combination of ESI-MS/MS and matrix-assisted laser desorption/ionization (MALDI)-MS/MS [22]. Two commonly used types of mass spectrometers regarding ion source in proteomic research are ESI and MALDI, which differ in the ways in which the proteins or peptides are ionized; each of them has certain advantages [23]. While MALDI is able to deal with complex mixtures and tolerate salts, ESI can be easily interfaced with separation strategies such as LC and CE. The singly charged ions produced in MALDI make it less complicated in terms of data interpretation compared with ESI in which multiple-charge states are generated for larger peptides. Typically, MALDI is coupled with mass analysis using a time-of-flight (TOF) mass analyzer, whereas ESI ionization is used as the ionization source associated with TOF, quadrupole, ion trap or hybrid mass analyzers [24]. High performance liquid chromatography (HPLC) coupled with ESI/MS or -MS/MS has detected the diversity of individual caseins [25]. Holland et al. [26] characterized glycosylation and phosphorylation sites in bovine k-caseins using 2-DE coupled with MALDI-TOF-MS and nano ESI-MS/MS. Most lactosylation sites on milk proteins in processed milk products have been characterized using MALDITOF- MS and nLC-ESI-LIT-MS/MS [27]. Recently, Guerrero, Lerno, Barile, and Lebrilla [28] have simultaneously detected different forms (genetic variants, phosphorylation and oglycosylation) of k-CN glycomacropeptides (GMP) by using ESIFTICR- MS, reporting for the first time glycoforms of GMP. MS/MS spectra of milk proteins are searched using the SEQUEST or Mascot search engine (either online or in-house server) against the Swiss-Prot database. Search parameters are often as follows: trypsin as a proteolytic enzyme, carbamidomethylation as a fixed modification, methionine oxidation, phosphorylation or glycosylation as variable modifications, etc. MS and MS/MS mass tolerances are of 0.1% and 0.5 Da, respectively. The spectra can also be validated manually if Mascot scores are below significance threshold. Certain peptide modifications can be manually assigned by visual spectra inspection [15,27].

Proteomes of dairy animals

During the last decade milk of various species, including human's, has been the center of proteomic research because of its importance in human's diet. Specifically, bovine milk (CM for cow milk) is the most studied of all as it has economic impact and is the most consumed kind universally. However the whole proteome and the low abundance proteins have yet not fully characterized in depth[29]. Lemay et al. [30] gathered publicly available data regarding milk proteome and mammary expressed sequence tags, leading to the identification of 197 milk protein genes and over 6,000 mammary genes in the bovine genome.D' Alessandro et al. [31] tried to present in brief the data and concluded to 573 non-redundant annotated protein entries referring to nutrient transport, lipid metabolism, and the immune response pathway.

Bovine and caprine milk contain about 2.1–3.5% proteins in comparison to equine and human milks with 2% and 0.8–1.5%, respectively. Bovine and caprine milks have a high casein to whey ratio of 5.0 and 6.0, whereas equine and human milks have a ratio of 1.5 and 0.9, respectively [11, 21, 24]. Nearly all caseins in milk are organized into colloidal particles known as casein micelles [11]. α_{s1}-, α_{s2}- and β-caseins are mainly located in the interior of the micelle and are bound to calcium phosphate nanoclusters by their phosphoserine domains [8, 23]. The proteins are also linked to each other by calcium bridges and hydrophobic interactions [23]. κ-Casein is found on the surface of the micelle with the hydrophilic C-terminal part protruding into the solvent [5, 23]. κ-Caseins are not evenly

 doi:10.20944/preprints202210.0097.v1

distributed on the surface, but are present in clusters making them vulnerable to proteolysis by enzymes such as chymosin.

The last few years, interest regarding proteins in low concentrations has risen, as many researchers suggest that these molecules may have crucial role in many physiological pathways. For that reason, an in-depth identification of milk protein components is needed in order to understand the complexity of milk proteins in the same or among species and their biological significance for the design of new products [32]. By coupling, Cunsolo and colleagues [32] using 1D SDS-PAGE with LC-MS/MS analysis identified 343 unique proteins in ovine milk [sheep milk(SM)], with the majority of them demonstrating catalytic or binding activity and involvement in metabolic or cellular processes or in physiological responses to pathogens, environmental or developmental stimuli.Furthermore, two studies aimed to an in-depth characterization of mature milk whey proteome from sheep have identified over 600 low-abundance proteins [33,34]. Specifically, Ha et al. [33] using the commercial CPLL kit followed by an in-gel digestion of a 1D-PAGE separation and an in-solution digestion followed by OFFGEL isolectric focusing fractionation, and LC-MS/MS analysis identified 669 proteins, mainly involved in immune response and regulation of inflammation. Using the two complementary proteomic ways, protein mixture digestion followed by LC-MS/MS analysis of peptides and 2-DE separation and MALDI-TOF/MS analysis, Anagnostopoulos et al. [34] investigated the whey milk of three Greek sheep breeds, identifying a mean of about 600 protein groups, most of which are involved in the nutrient transport and in the immune system response. Also, Tomazou and colleagues [35] in a descriptive study of sheep and goat milk found 84 common antimicrobial peptides (AMPs) in both species in all the tested breeds, whereas the caprine milk presented the greatest diversity in unique AMPs.

A comparison of unique gene products between sheep and cow's milk revealed that the two proteomes have in common 434 proteins (about 40% of cow milk (CM) proteome), sheep milk (SM) contains 1077 unique components, whereas CM 653.. GO analysis of common proteins and those identified exclusively in sheep's and cow's proteomes demonstrated that the majority of shared components are "enzyme modulators" and "hydrolases" presenting catalytic and binding activity and are involved in cellular, metabolic and localization processes [32].

A significant number of health-promoting properties, including regulation of the immune, cardiovascular, nervous, and gastrointestinal systems are attributed to peptides and their precursors originated from bovine milk[36]. On the other hand, many of CM proteins are suggested to promote allergies in human and have greater allergenic potential than other milk proteins originated from other species including small ruminants. Milk protein composition is reported to vary within species, arising from differences in physiology and the varying nutritional requirements of offspring. Major proteins in sheep milk have been shown to possess differences in amino acid composition in comparison to those of cow milk [33,37]. According to Ha et al. [33], who compared the proteomes from ovine and bovine milk, the unique peptides of cow whey are involved in cellular growth and metabolism, whereas those in sheep are participating in cellular establishment, signaling, protein maturation, and inflammatory and other immune responses. GO analysis of proteins commonly identified in both species revealed these proteins are involved in immune response. In ovine milk whey proteins of the complement system C1QA, C1QB, C1QC, and C8A, together with CRP, KLKB1, KRT1, MASP2, TLR4, and YWHAZ are associated with acute inflammation and defense response; proteins absent in bovine milk whey.

Specifically,, about 71% (i.e. 1077 of 1511 unique gene products) of SM proteins seemed to be unique to ovine species including components with potential benefits for human health. The list of proteins identified exclusively in the SM proteome contained several constituents (i.e. Myeloid antimicrobial peptide, MAP34; Bactenecin 6, Bac6; Bactenecin 7.5, Bac7.5; and Bactenecin 11, Bac11; Cathelicidin, CATH2; Cathelin-related pep-

 doi:10.20944/preprints202210.0097.v1

tide, CATHL5; Cathelicidin-7, CATHL7 and Coagulation factor XII F12) playing a physiological role in the immune response to pathogens. Some examples of proteins with health beneficial are the ADM2 protein involved in cardiovascular homeostasis; the Angiopoietin-like protein 4 (ANGPTL4) a possible pivotal modulator of cell-cell junctions and vascular integrity with a protective role in myocardial infarction [38]; the Alphaaminoadipic semialdehyde dehydrogenase (ALDH7A1) a participant in detoxification of aldehydes generated by alcohol metabolism and lipid peroxidation; the Peripilin (ADFP) linked to lipid globule surface membrane material and probably involved in development and maintenance of adipose tissue and other tissues, including the lactating mammary gland; so that it may serve as a marker of lipid accumulation in diverse cell types and diseases; the S100A3 protein a potential protective molecule of hair from oxidative damage due to very high Cys content [39]; and, finally, a group of ribosomal proteins (i.e. L6, L23, S3 and S7) exerting key role in suppressing tumor cell proliferation [40].

Proteins and related peptides derived from CM can be categorized into four groups: caseins (aS1-, aS2-, b- and k-caseins), serum proteins [a-lactalbumin (a-La), b-lactoglobulin (b-Lg), bovine serum albumin (BSA), immunoglobulins (Igs) and a range of other minor whey proteins], proteose peptones (low molecular weight peptides derived from caseins and proteose peptone component 3, called PP3) and membrane [mostly milk fat globule membrane (MFGM)] proteins [15]. Among the serum and the MFGM proteins , there is a range of enzymes; components of several enzyme systems are linked with casein (CN) micelles. The somatic cell fraction is considered to be part of the milk profile and contains a variety of enzymes and other molecules that may alter the milk proteome. The origin of somatic cells enzymes can be from already dead cells which release their components into milk or from live somatic cells from the mammary gland [41]. PTMs significantly increase the complexity of the milk proteome. Many proteins are under a multitude of co- and post-translational modifications, including phosphorylation, acetylation, glycosylation, disulphide crosslinking, conjugation with lipids and proteolytic cleavages, resulting in a variety of changes in milk protein structure and functionality [42,43].

In-depth proteomic analyses of milk from individual cows revealed that even milk from a single animal shows different phosphorylation variants of each individual casein (CN) regarding number of modifications per molecule [44,45]. Recent genomic studies are trying to elucidate the factors causing this variation. Bijl et al , suggesting [46] have found that for aS1-CN-8P and aS1-CN-9P high heritability is present but the genetic correlation is low implying different genetic background and regulation but a strong genetic influence. In the same study, aS2-CN-11P and 12P were relatively highly correlated in two breeds, Danish Jersey and Danish Holstein, which implyed more common genetic influence. It was pointed out that the 8 common phosphorylation sites of the aS1-CN-8P and -9P occur in the S-x-E/pS motif, and the extraphosphorylation site of the aS1-CN-9P on Ser56 occurs in the S-x-D motif. It was concluded that the extra phosphorylation site of aS1- CN-9P is phosphorylated by a kinase different from the one phosphorylating the more common S-x-E/pS motif resulting in the major aS1-CN-8P molecular pool [46]. Proteomic analyses using LC-ESI-single quadrupole MS have further shown that the fraction of aS1-CN-9P and aS2-CN-12P relative to lower phosphorylation forms were higher in poor- and non-coagulating milk [47], indicating a clear influence of phosphorylation degree on functional properties. Furthermore, breed differences, with Danish Jersey having higher relative contents of less phosphorylated forms of aS1-CN (aS1-CN-8P) and of aS2-CN (aS2-CN-11P) compared with Danish Holstein using the same method, have been shown [48]. This work also revealed the effects of lactation stage on degree of phosphorylation of both aS1- and aS2- CN. Regarding cleavage by chymosin, a recent study showed that 15% more hydrolysis of aS1-CN-8P into aS1-CN f1e23 and f24e199 occurred in milk, while no difference was observed in reconstituted sodium caseinate solutions [15,49].

The major differentiating proteins for human and ruminants milk were lactotransferrin (LTF), β-casein (CSN2), serum albumin (ALB), osteopontin (SPP1), bile salt-activated lipase (BSSL) and α-lactalbumin (LALBA) which are higher in abundance in human

 doi:10.20944/preprints202210.0097.v1

milk, whereas, β-lactoglobulin (LGB) and glycosylation-dependent cell adhesion molecule 1 (GLYCAM1) in ruminants' milk [50]. Whey proteins may exert a critical role in the process of proteolysis. The amount of proteins related to proteolysis, regulation of signal transduction, immune response, biological adhesion, regulation of cell differentiation, and cytokine production were higher in human milk. In goat milk, proteins associated to cell mobility were found in higher concentration, whereas in cow milk were found the ones related to lipid localization The majority of whey proteins were secreted and originated from cellular organelles, including lysosome, melanosome, endoplasmic reticulum lumen and Golgi lumen; and demonstrated affinity with nutrients such as amino acids, lipids, calcium and vitamins. Furthermore, these proteins exibited peptidase, antioxidant and cytokine activity. The intensity of proteins with carbohydrate derivative binding, peptidase activity, glycosaminoglycan binding, lipid binding, and cytokine activity were found in increased levels in human milk. However, proteins with enzyme binding were found in great concentration in goat milk according to Lu et al [50]. A significant amount of complement proteins and regulators were also detected in goat milk, including C9, C7, C3, C4BPA(C4b-binding protein alpha chain), C1S , CFI (complement factor I), A2M (α-2-macroglobulin) and SERPINF1 (pigment epithelium-derived factor). Two major acute phase proteins, SAA1 and SAA3, were expressed in higher abundance in goat milk. In general, antimicrobial proteins were found in higher concentration in ruminant's milk. As for the family of cathelcidins, Cathelicidins 1 and 2 were more expressed in goat milk, in comparison to cathelicidins 4 and 6 which were expressed in cow milk. LPO,proteins with antimicrobial properties, were in higher concentration in cow and yak milk [50].

The huge amount of antimicrobial proteins in ruminants milk can be attributed to the fact that ruminant's placenta has a barrier for the majority of proteins including immunoglobulins and others with antimicrobial properties and for that reason the neonatal can only be protected via colostrum and milk. Simultaneously, the mammary gland must be protected from bacterial infection [30].

The most shared proteins across species were involved in protein/vesicle-mediated transport, along with major MFGM proteins such as BTN, ADPH, FABP, and MUC1. The main difference regarding human MFGM proteome was a higher enrichment in enzymes involved in lipid catabolism, also reported in Liao et al. [51] and in a set of immune response proteins [52,53]. In 2015, another research team compared the proteome of cow, yak, buffalo, goat, and human MFGM [54]. The authors identified a total of 520 proteins of all species, most of which were shared among all species, although in different isoforms, as also reported by other studies [51,55], such as BTN, lactadherin, MUC1, and ADPH. These findings showed that the MFGM proteome presents a high complexity and variability among species. In terms of molecular function and Gene Ontology (GO) categories, cellular process, localization, transport, signal transduction, and response to stimulus were enriched in all the MFGM fractions [54]. Comparative proteomic analyses have been performed to compare cow and goat proteome. In a 2019 study, a total of 776 MFGM proteins were identified: 427 and 183 that are unique for goat and cow milk, respectively, and 166 proteins shared between the two species. Most of the goat MFGM proteins were related to metabolic processes (about 21%), whereas most of the cow MFGM proteins were related to disease-associated pathways (about 49%) [56]. Subsequently, the same authors evaluated the variations between goat colostrum and mature MFGM proteome. They found a higher number of proteins than in their previous study; in particular, 543 and 858 proteins in colostrum and mature milk, respectively, of which 394 are shared in colostrum and mature milk. Colostrum was found to have fewer proteins but more functions of protein processing in the ER than mature milk, whereas mature milk had more metabolism-related proteins [57]. Yang et al. investigated the variation among species by analyzing MFGM glycoproteome from cow, buffalo, yak, goat, and human milk. They found that the glycoproteins from the different MFGM species were mainly related to the response to stimulus, according to the GO categories, and that the fractions from ruminants (cow,

 doi:10.20944/preprints202210.0097.v1

buffalo, yak, goat) were more similar to each other when compared to the non-ruminant's fraction (human) [54,58,59].

MFGM

The milk fat globule membrane (MFGM) is a surface-active membrane derived from the apical membrane of the secretory cell in the mammary gland [60]. It surrounds each of the milk fat globules (MFG) allowing them to remain dispersed in milk. The MFG core is essentially composed of triglycerides, while the MFGM envelope is a true polar lipids bilayer with proteins, enzymes, neutral lipids and other trace elements [61]. Phospholipids and glycoproteins from the MFGM affect several cell functions, such as growth, molecular transport system, memory processing, stress responses, and central nervous system myelination [62]. In milk, the MFGM enables the fat to remain dispersed throughout the aqueous phase. In addition to this role, some human MFGM proteins, such as secretory immunoglobulin (Ig), mucins and lactadherin; have been reported to play an important part in various cell processes and defense mechanisms against bacteria and viruses in the newborn [63-66]. The antimicrobial defense of human, horse and donkey milk is based on lysozyme and lactoferrin systems, in contrast to cow milk where lactoperoxidase and the Igs are the defensive mechanisms [1].

MFGM components can serve as a target for bacterial adhesion [67,68]. Adherence of bacteria to intestinal epithelial cells is considered an important step for colonization by different pathogenic and nonpathogenic microorganisms [69]. Salmonella enterica serovar Enteritidis (Salmonella Enteritidis) is an important foodborne enteric pathogen that has been shown to use specific mechanisms of molecular binding to both biotic and abiotic surfaces. MUC-1 (mucin 1), which is a major MFGM protein, has recently been shown to bind gram-negative bacteria in vitro [70,71]. In vivo trials using BALB/cA mice have shown that bovine milk glycoconjugates may play a role in the inhibition of Helicobacter pylori infection. It was also reported that milk fat globules, MFGM as well as the glycosphingolipid lactosylceramide and the gangliosides GM3 and GD3, were able to inhibit the binding of enterotoxigenic E. coli strains and prevent their hemaglutination [67,72]. In a study conducted by Guri et al., [67], they found that the exposure of the cells (human cancer cell line HT-29) to fat globules before the infection inhibited the internalization of Salmonella. It has been reported [68] that washed MFGM from sow milk significantly inhibited the adherence of K88-positive Escherichia coli to the intestinal brush border membranes and that the fat globules may adhere bacteria through their surface Ig. The latter study showed that the milk fat globules originating from bovine and goat milk can inhibit the binding and internalization of the foodborne pathogen Salmonella Enteritidis to HT-29 colon cancer cells.

Butyrophilin belongs to MFGM proteins and is found in great concentrations. To that protein has attributed the modulation of encephalitogenic T-cell response to myelin oligodendrocyte glycoprotein in cases with experimental autoimmune encephalomyelitis related to human\ multiple sclerosis [73,74]. Fatty acid binding protein has been found to have anticancer effect on breast cancer cell line [75,76]. Mucins are another antibacterial family known for their anti-adhesive property because of their high glycosylation protecting the epithelial cells from enzyme and bacteria attacts [70,71]. Vesicle mediate transport is the way that the major milk components, including proteins, lactose, phosphate, citrate, calcium, and in some opinion as well as lipids, are transported and finally secreted into milk. Conserved proteins across species were mainly involved in the process of protein/vesicle mediate transport, which could be explained by the origin of MFGM from the intracellular membrane and plasma membrane of epithelial cells in mammary gland [77]. Proteins with involvement in secretion of lipids to milk, such as ADFP, BTN1A1, XDH and MFGE8, can also be found in MFGM across species. ADFP, BTN1A1 and XDH formed a tripartite structure to secret lipid droplets in plasma membrane of epithelial cells. MFGE8 also contributed to MFG secretion [78]. Lu et al [52] found that the major proteins in MFGM are conserved across species. The abundance of these proteins however

 doi:10.20944/preprints202210.0097.v1

was different. XDH and stomatin, which accounted for 25% and 3% in goat MFGM, were significantly higher in abundance than those in other MFGM. Unlike in other MFGM with BTN1A1 as the most abundant proteins, XDH was observed to be the most abundant proteins in goat MFGM which was in consistency with previous studies [79]. XDH is a redox/purine catabolizing enzyme. In MFG, it is considered to cooperate with BTN1A1 for MFG secretion. The ratio of XDH/BTN1A1, the two most abundant MFGM proteins usually varied within species [80,81]. Cathelicidins were uniquely present in cow MFGM. They belong to a family of proteins with antibacterial activity by binding bacterial lipopolysaccharides. There is a hypothesis regarding cathelicidins origin according to which the specific proteins come from neutrophilsand they have been identified in MFGM in previous proteomics studies [82],indicating the expression of them in epithelial cells in mammary gland. The presence of cathelicidin in milk could be due to their antibacterial properties. Also, in studies conducted by Katsafadou et al [83-85] in ewes with mastitis caused by Mannheimia haemolytica, cathelcidin-1 was found to be an early mastitis index in ewes by means of 2D-gel electrophoresis followed by MALDI-TOFF analysis. In the same experiments an association of number of somatic cells in ovine milk with the concentration of cathelcidin-1 in blood plasma was revealed, since the increased number of somatic cells in milk is marker of subclinical/clinical mastitis in ruminants. Another antibacterial proteins found in cow MFGM was peptidoglycan recognition protein 1, which kills gram-positive bacteria by binding their peptidoglycans and interfere peptidoglycan biosynthesis [86].

To the MFGM originated from ruminants' milk, especially bovine, are attributed health benefits including anti-carcinogenic, antimicrobial, anti-inflammatory, and anticholesterolemic effects [87,88]. Three researchers groups tested the anti-carcinogenic activity of MFGM on HT-29 cells (a human colon cancer cell line) [89-91], proving the reduction of proliferation and enhancement of apoptosis of the cancer cells through effector caspase-3 activation. The antimicrobial activity was assesed through the inhibition of in vitro rotavirus infectivity [92] and the anti-adhesive activity exerted by a mucin 1 (MUC1)-enriched MFGM fraction against bacteria in the gut mucosa [93]. The anti-inflammatory activity was observed with the in vivo mitigation of lipopolysaccharide (LPS)-induced intestinal damage and inflammation in low birth weight (LBW) mice [94] and with the decrease of pro-inflammatory serum markers such as total cholesterol, low density lipoprotein (LDL)-cholesterol, along with an increased production of anti-inflammatory cytokines in obese adults challenged with a high-fat meal rich in saturated fatty acids [95]. Finally, the anticholesterolemic activity was evaluated by the decrease exerted by MFGM-derived sphingomyelin of the intestinal absorption of cholesterol and fats in animal models, thus protecting the liver from fat- and cholesterol-induced steatosis and consequently preventing the inflammatory condition involved in atherosclerosis and insulin resistance [88,96]. Concluding, MFGM may exhibit a key role in prevention of health conditions as part of nutraceutical products [59]. The most common MFGM proteins are adipophilin (ADPH), butyrophilin (BTN), mucin 1 (MUC1), xanthine dehydrogenase/oxidase (XDH/XO), CD36, periodic acid Schiff III (PAS III), PAS 6/7, lactadherin, and fatty acid-binding protein (FABP) [88,97,98]. ADPH, also known as perilipin 2, is a major constituent of the MFGM localized in the inner monolayer membrane and regulates lipolysis by controlling the access of proteins to the MFG [97,99]. BTN is a transmembrane protein and is the most abundant protein in bovine MFGM. BTNs are members of the immunoglobulin (Ig) superfamily, and BTN1A1 is the form in human MFGM [97,99,100]. Studies have shown that the knockout of BTN1A1 in bovine mammary epithelial cells decreased the size and the phospholipid content of lipid droplets (the precursors of MFGs), implying that BTN1A1 has an active role in regulating the synthesis of lipid droplets via a mechanism involving membrane phospholipid composition [101]. MUC1 is a glycoprotein with highly glycosylated extracellular domains localized on the outer surface of MFGs. The latter enhances its resistance to digestion and potentially available to act as a decoy receptor for pathogens [93,97,99]. XDH/XO is a redox enzyme that accounts for 12% of total

 doi:10.20944/preprints202210.0097.v1

bovine MFGM proteins and is localized in the intermembrane space between the monolayer and the bilayer, forming a tripartite structure with BTN and ADPH. It plays a role in antimicrobial defense of the gastrointestinal (GI) tract through the production of reactive oxygen species (ROS) and reactive nitrogen species (RNS), which have bactericidal properties. Surface carbohydrates and XDH/XO may possibly act as decoys—pathogens can interact with receptors of the epithelial cells of the GI tract, but can also bind to similar receptors on the MFGM that themselves can act as decoys, such as MUC1 [93], to avoid bacterial interaction with their primary target (GI epithelial cells) and that can also impart an antimicrobial effect thanks to ROS/RNS production by XDH/XO [88,99,100]. Finally, FABP is a protein with a similar localization of XDH/XO and plays a key role in the synthesis of MFG lipid constituents during the intracellular transport of FAs. Indeed, it is involved in the transport of FAs through the capillary endothelium to reach the cytoplasm of mammary endothelial cells, where they cross the membrane via diffusion [99,102]. Some studies [88,103] have shown the presence of the onco-suppressors breast related cancer antigens 1/2 (BRCA1 and BRCA2) in human and bovine MFGM. These two oncosuppressors are involved in DNA repair processes [88]. Their presence in the MFGM may be explained because MFGs are secreted by mammary gland epithelial cells and carry a fraction of their apical plasma membrane. The nutraceutical role of the MFGM and its potential anticancer effect could be based on the fact that after MFGM consumption, the inhibitory peptides might be released from MFGM and subsequently absorbed by the digestive tract. The absorbed peptides could enter the bloodstream and reach the organs (or tissues), where they inhibit the transforming cells [87]. Ji and collaborators [91] evaluated the antiproliferative effect of five MFGM fractions from yak, bovine, goat, camel, and buffalo milk using the HT-29 cell line. The antiproliferative effect was evaluated in terms of cell viability, cell cycle, cytomorphology, apoptosis, and mitochondrial membrane potential (MMP). The results revealed that all the five MFGM fractions reduced cell growth by affecting cell cycle and inducing apoptosis, whereas MMP values were also significantly reduced by all the five MFGM fractions. Among all the tested samples, buffalo and goat MFGMs were more effective in inducing apoptosis than the other three MFGMs leading to the assumption that MFGM may be used for human colon cancer prevention [59,91].

Bioactive and allergens

Many studies have proved that milk holds proteins with significant bioactive properties, especially when they are digested by enzymes in human's gastrointestinal tract and bioactive peptides are released [36]. The latter has grown the need for products enriched to those peptides as well as the discovery of them [104,105], a field where proteomics techniques can be applied and found useful [33].

As bioactive peptides can be addressed the constituents (genuine or generated) of ready-to-eat foods which may exhibit a regulatory activity in the human organism, irrespective of their nutritive functions [106,107]. Milk proteins are considered to contain a great amount of bioactive peptides encrypted within the primary structure of milk proteins, needing proteolysis for their release from precursors during digestion or food processing. Digestive enzymes, naturally occurring enzymes in milk, coagulants and microbial enzymes, especially from adventitious or starter lactic acid bacteria, generate bioactive peptides during milk fermentation and cheese maturation, thereby enriching the dairy products [108]. In general, the rules followed for the identification and characterization of bioactive peptides are isolation from in vitro or in vivo enzymatic digestion of precursor proteins or and chemical synthesis based on combinatorial library designs of peptides that have a structure identical to that of those known to be bioactive [107,109].

Several milk-derived peptides reveal multifunctional properties; for example, specific peptide sequences having two or more different physiological activities. Some regions in the primary structure of caseins (CN) contain overlapping peptide sequences that exhibit different activities. These regions have been pointed out as 'strategic zones' partially protected from further proteolytic breakdown [110]. Milk protein-derived bioactive

 doi:10.20944/preprints202210.0097.v1

peptides can act as regulatory substances and are characterized as exorphins or formones (food hormones) due to their similarity to opium (morphine) and naloxone inhibiting properties [106,111]. The α – and β -casomorphins and lactorphins act as opioid agonists, while casoxins act as opioid antagonists [111]. Casomorphins may produce analgesia, modulate social behavior, influence postprandial metabolism by stimulating the secretion of insulin and somatostatin, and influence gastrointestinal absorption [111]. The common structural feature among endogenous and exogenous opioid peptides is the presence of a Tyr residue at the amino terminal end and the presence of another aromatic residue, Phe or Tyr, in the third or fourth position. This is an important structural motif that fits into the binding site of the opioid receptors [107]. Similarities between the clotting of blood and milk and between κ -CN and the human fibrinogen γ -chain have been found [110]. The dodecapeptide of fibrinogen γ - chain and the 106–110 sequence of the κ -CN and related sequences demonstrate functional homologies [110]. The casoplatelin, a peptide derived by trypsin hydrolysis of κ -CN exhibits antithrombotic activity by inhibiting fibrinogen binding to platelets. Casoplatelins also contain part of the κ -CN 106–169 sequence, the macropeptide fragment, which inhibits gastrin and then acid secretion in the stomach [106]. The majority of immunomodulatory peptides are hydrolysate derivatives of major milk proteins [112]. a_{s1} – and β -CN-derived immunopeptides stimulate phagocytosis of sheep red blood cells by murine peritoneal macrophages and exert a protective effect against Klebsiella pneumoniae infection in mice after intravenous administration of peptides [113]. The C-terminal β -CN sequence 193–209 containing β -casokinin decapeptide prompted a significant proliferative response in rat lymphocytes [114]. Recently, researchers have focused on immune-enhancing properties of caseinophosphopeptides (CPPs) and whey-derived peptides [107]. The immunomodulatory activity of CPPs was attributed to phosphoseryl residues even though the phosphorylation site appears to be an allergenic epitope in CN [115].

Bioactive peptides

Milk has many putative health-promoting benefits over and above simple nutrition [116]. A number of studies have correlated the consumption of milk peptides with lower blood pressure, as reviewed by Haug et al. [117]. Bioactive peptides encoded within milk proteins can also exhibit antihypertensive, antithrombotic, immune-stimulatory or antimicrobial effects [118,119]. ACE (peptidyl-dipeptidase A; EC 3.4.15.1) is a multifunctional ectoenzyme located in different tissues and exerting a key physiological role in many axes such as renin–angiotensin, kallikrein–kinin and immune systems. The enzyme is responsible for the increase in blood pressure by converting angiotensin I to the potent vasoconstrictor, angiotensin II, and by degrading bradykinin, a vasodilatatory peptide, and encephalins [120]. For that reason, ACE inhibition mainly results in a hypotensive effect but may also influence different regulatory systems involved in immunodefence and nervous system activity [106]. ACE-inhibitors derived from milk proteins represent different fragments of CN, named casokinins, or whey proteins, named lactokinins [106]. Studies have stated an inverse association between consumption of unprocessed cows' milk and respiratory infections [121]. It has also been noticed that pasteurised milk, boiled farm milk and raw farm milk but not UHT milk exerted an independent protective effect on fever [121]. GLCM1 is a sulphated glycoprotein in the mucin group of proteins [122]. Mucins play a role in providing immune protection to infants from pathogens [122], which highlights the potential importance of this protein in infant formula products [119].

Sheep milk whey proteins recently are gathering research interest for their bioactivity or health beneficial, such as immunomodulatory, antimicrobial and transfer of passive immunity [123]. In addition, natural digestion of sheep whey proteins in the gastrointestinal tract can generate peptides with various bioactivities, such as antihypertensive, opioid, antibacterial, antioxidant, and immunomodulatory activities [33,124,125]. Xanthine dehydrogenase/oxidase acts as a converter of hypoxanthine to xanthine in most cell types.

 doi:10.20944/preprints202210.0097.v1

However, investigation of expression of xanthine dehydrogenase/oxidase in the mammary gland implied that the function of this enzyme in milk is likely to reverse the endocytotic process of milk fat globule secretion [126].

The largest group of goat unique proteins includes constituents that are precursors of peptides involved in possible hypotensive effects (i.e. angiotensinogen); or mediate inflammation and tumor progression (i.e matrix metalloproteinase); or have involvement in mammary gland development (i.e. dystroglycan); or have a key role in fetus' immunological protection (factor H) or act as defensive molecules(complement component C2 and C6). Furthermore, the identification of proteins belonging or related to complement system (vitronectin, factor H, fibulin-1, peroxiredoxin 2) confirmed the anti-inflammatory properties of goat milk and its function in the regulation of immune response for the maintenance of immune homeostasis [127]. In brief the bioactive peptides are described in Table 1.

Peptide/Protein	Biological function	Reference
α-casomorphin	opioid agonist	[111]
β-casomorphin	opioid agonist	[111]
lactorphins	opioid agonist	[111]
casoxins	opioid antagonist	[111]
κ-casein	milk-blood clotting factor	[110]
casoplatelin	antithrombotic agent	[106]
macropeptide fragment	gastrin inhibitor	[106]
α_{s1} casein immunopeptides	phagocytosis stimulator	[113]
β casein immunopeptides	phagocytosis stimulator	[113]
β-casokinin decapeptide	proliferation stimulator in lymphocytes	[114]
caseinophosphopeptides	immunomodulatory activity	[102]
casokinins	ACE inhibitor	[106]
lactokinins	ACE inhibitor	[106]
GLCM1	infants' immune protection against pathogens	[122]
xanthine dehydrogenase/oxidase	antioxidant properties	[126]
angiotensinogen	hypotensive effect	[127]
matrix metalloproteinase	inflammation/tumor progression modulator	[127]
dystroglycan	mammary gland development	[127]
complement component C2	defense factors	[127]
complement component C6	defense factors	[127]
factor H	immunological protection of the fetus/anti-inflammatory properties/regulator of immune response	[127]
vitronectin	anti-inflammatory properties/regulator of immune response	[127]
fibulin-1	anti-inflammatory properties/regulator of immune response	[127]
peroxiredoxin 2	anti-inflammatory properties/regulator of immune response	[127]

Peptides demonstrating antimicrobial properties

The natural defensive systems of milk consist of immunoglobulins, predominantly secretory immunoglobulins, and other components such as lysozyme, lactoferrin, the lac-

 doi:10.20944/preprints202210.0097.v1

toperoxidase system, macrophages, and lymphocytes [128,129]. Biodefensive peptides derived from milk proteins such as casein, α-lactalbumin, β-lactoglobulin, and serum albumin have been reported [128,130-132]. The generation of those peptides occur during digestion after enzymatic hydrolysis by pepsin (in the stomach) and/or pancreatic and intestinal proteases, such as trypsin, chymotrypsin, and peptidases [129]. Sequence identity between regions of α-lactalbumin and lysozyme has been reported [133,134], and a great number of studies propose that antipathogenic milk peptides are produced in vivo during digestion, especially from whey and casein protein substrates [128,130,132]. In one example, several tryptic peptides derived from β-lactoglobulin with associated antibacterial activity against gram-positive microorganisms such as Bacillus subtilis [132] were somewhat analogous to regions contained within the structure of bovine butyrophilin, a major structural protein associated with the MFGM. Thus, antibacterial activity observed in some of these MFGM hydrolysates may result from the generation of similar and/or equivalent biodefensive peptide sequences.

The latter may due to the synergistic activity of naturally occurring proteins and peptides, in addition to peptides generated from inactive protein precursors [130]. In mammals, antimicrobial peptides are found at the epithelial surfaces and within granular phagocytic cells and constitutes of innate defenses, due to their ability to kill microorganisms and modulate inflammatory responses [135]. Lactenin was probably the first antibacterial factor derived from milk and the nature or origin of this inhibitor of streptococcal growth was not elucidated, the antibacterial activity was found to remain together with the whey protein fraction after rennet treatment, and the active component appeared to be resistant to tryptic hydrolysis. A group of basic, glycosylated and high-molecular weight (~5 kDa) polypeptides, called casecidins, are released from chymosin-treated CN [106]. These peptides demonstrate bactericidal properties against lactobacilli and other bacteria such as Staphylococcus aureus. Another antibacterial peptide derived from αs1-CN treated with chymosin, called isracidin, which corresponded to the N-terminal fragment of this protein (f1–23), was isolated [106]. Isracidin is a 23-mer peptide that contains 6 basic residues, including 5 in the N-terminal region, and 7 hydrophobic residues, with the net charge 2.2 [136] and is an inhibitor of the in vitro growth of lactobacilli and other Gram-positive bacteria when they are found on relatively high concentrations (0.1–1 mg/mL). However, in vivo, isracidin exerted a strong protective effect against S. aureus, Streptococcus pyogenes and Listeria monocytogenes when administered at doses as low as 10 μg per mouse prior to bacterial challenge. This peptide also safeguarded sheep and cows against mastitis when injected into the udder at levels comparable to those observed with standard antibiotic treatment. Bovine α_{s2}-CN was also shown to be a precursor of several peptide fragments with antibacterial activity. A positively charged peptide, corresponding to αs2-CN f150–188isolated from boiled and acidified milk and called casocidin-I [137], demonstrated inhibitory activity against Gram-negative (Escherichia coli) and Gram-positive (S. carnosus) bacteria. Subsequently, the antibacterial role of α_{s2}-CN was re-evaluated with the objective of verifying the existence of an enzymatically induced antibacterial domain [125]. Hydrolysis of α_{s2}-CN with the gastric enzyme pepsin rendered two different antibacterial peptides: one, f164–179, which was included in the casocidin-I, and a new antibacterial fragment at the C-terminus of the protein, f183–207. The latter had a consistently higher activity than f164– 179, although both peptides showed a comparable hemolytic effect. It has also been proposed that part of the antibacterial activity of human milk resides in the CN fraction, particularly in glycosylated κ-CN and its glycosylated region, the caseinomacropeptide [138]. Another peptide called kappacin, corresponded to nonglycosylated, phosphorylated bovine caseinomacropeptide (κ-CN f106–169), which showed growth-inhibitory activity against Gram-positive (Str. mutans) and Gram-negative (Porphyromonas gingivalis) bacteria. Additionally, it exhibited the capacity to bind enterotoxins and to inhibit viral and bacterial adhesion, e.g. the binding of cariogenic bacteria to oral surfaces. Lactoferricin, an antibacterial peptide isolated from a peptic hydrol-

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ysate of bovine and human lactoferrin (f17–41 and f1–47, respectively), demonstrated inhibition against a wide variety of microbial species, including many important foodborne pathogens such as L. monocytogenes [139]. Lactoferricin exhibited higher bacteriostatic and bactericidal activity compared to intact lactoferrin. Proteolytic digestion of bovine β-lactoglobulin by trypsin yielded four peptide fragments with bactericidal activity. These peptides corresponded to β- lactoglobulin f15–20, f25–40, f78–83 and f92–100 [132] andexerted bactericidal effects against Gram-positive bacteria only.

Molecules with antimicrobial properties should be used in the development of new antimicrobial treatments, new natural food preservatives or nutraceuticals [140], especially when they are combined and have higher effectiveness such as the system of lactoferrin, lactoperoxidase and lysozyme [130]. Antimicrobial milk constituents may present antibiotic-like activity and substitute antibiotics [141]. Moreover, the milk proteins' sequences contain several motifs that can be released during enzymatic hydrolysis to increase antimicrobial potential of milk proteins [142]. Subjected to proteolytic conditions, a diverse combination of bioactive peptides deriving from milk proteins can be obtained, but only few peptides have been identified and characterized for their antimicrobial activity [143-150]. In addition, several milk protein-derived peptides exhibit more than one type of activity, and they are referred to as multifunctional peptides [150,151]. Proteins playing a physiological role in the defense/immunity mechanisms or in the nutrient delivery system were discovered, such as proteins operating in the nutrient delivery mechanism include the ATP synthase subunit alpha (gene code: ATP5A1), Desmocollin-1 (DSC1), an uncharacterized protein (UniProt Acc. No. W5P1W2) similar to the Folate receptor 3-gamma (FOLR3) and two Ras-related proteins Rab4b and Rab- 35. Among the proteins playing a physiological role in the defense/immunity mechanism some components were found (i.e. Myeloid antimicrobial peptide, MAP34; Bactenecin 6, Bac6; Bactenecin 7.5, Bac7.5; and Bactenecin 11, Bac11; Cathelicidin-7, CATHL7) belonging to the family of cathelicidins, a large group of polypeptides, varying in amino acid sequence, structure and size, showing a broad spectrum of antimicrobial activity against bacteria, enveloped viruses and fungi [152]. In a study conducted by Cunsolo et al [32] were discovered proteins involved with inflammatory responses, include an uncharacterized protein (Acc. No. W5P4C6) similar to the Coagulation Factor XII F12 participating in stimulating inflammation, a normal body response to infection, irritation, or other injury; and the S100A9 protein, a calcium- and zinc-binding protein which plays a noticeable role in the regulation of inflammatory processes and immune response, and may also act as a potent amplifier of inflammation in autoimmunity as well as in cancer development and tumor spread. More than 80% of all peptides contain Lys, Gly and Leu amino acids. The Ile, Val, Ala, Arg, Ser, Phe, Asn, Thr, Gln and Pro are present in at least 50% of peptides, while Asp, Cys, Glu, His, Met, Trp and Tyr are their minor components. The content of amino acids in the sequences of the AMPs was calculated in reference to all of the examined peptides. The results indicate that certain amino acids are more common in the peptide sequences and can influence their biological activity. Amino acids such as Arg, His, Lys, Phe, Tyr, Trp, Leu, Pro could be predominant in the sequences of biologically active peptides, depending on their type of activity [153]. The analysis of amino acid content of antimicrobial peptides revealed many fragments with the predominance of one or several amino acids [154,155]. In brief the proteins/peptides with antimicrobial properties are described in Table 2.

Peptide/Protein	Antimicrobial properties	Reference
butyrophilin	Inhibitor of Gram$^+$ bacteria	[132]
lactenin	Inhibitor of Streptococcus spp growth	[106]
isracidin	Inhibitor of Gram$^+$ bacteria	[136]
casocidin-I	Inhibitor of Gram$^+$ and Gram$^-$ bacteria	[137]
kappacin	Inhibitor of Gram$^+$ and Gram$^-$ bacteria/viral/bacterial adhesion	[138]

 doi:10.20944/preprints202210.0097.v1

lactoferrin	Inhibitor of foodborne bacteria, Listeria monocytogenes	[139]
lactoferricin	Bacteriostatic, bactericidal activity	[139]
desmocollin-1	Defense/immunity stimulator	[152]
Ras-related proteins	Defense/immunity stimulator	[152]
cathelcidins family	Inhibitor of bacteria, enveloped viruses, fungi	[152]
coagulation factor XII F12-like	Inflammation stimulator	[32]
S100A9-protein	Regulator of inflammatory response	[32]

Peptides with allergenic potential

The high availability and consumption of bovine milk world widely have risen the problem of allergies. Between 5% and 15% of infants show symptoms suggesting adverse reactions to CM proteins [156], while estimates of the prevalence of cow's milk protein allergy (CMPA) vary from 2% to 7.5%. According to many clinical trials [157,158], the best substitute is donkey's milk (DM) presenting high resemblance to human milk (HM), with similar lactose and mineral contents, fatty acid and proteins with antibacterial properties, digestive activity molecules and growth factors and hormones [11,159]. The relationship between hypo-allergenicity of DM and its proteome fraction has been thoroughly examined by Cunsolo et al. [29]. According to them 106 unique products in this biological fluid, among which 10% could be ascribed to the donkey, 70% were homologous to Equus caballus and only just about 3% to bovine milk [160]. Concluding, milk proteins from donkey and Bos taurus share low-sequence similarity [161-163]. Particularly, sequence alignment of donkey's αs1- and αs2-CNs (caseins) with their bovine counterparts (i.e. two of the major cow's milk allergens) highlighted that the IgE-binding linear epitopes of both cow's αs-CNs and the corresponding domains present in donkey's counterparts had remarkable differences in their amino acid sequences, which could be related to the already demonstrated low allergenic properties of DM.

Different milk proteins (e.g. α-lactalbumin, serum albumin, lactoferrin) can promote an allergic reaction, but casein fractions and β-lg are the most common milk allergens [164]. B-lg is absent from human milk and has not been detected in camel and llama milk, but is present at relatively high concentrations in bovine, buffalo, sheep and goat milk as well as in horse and donkey milk. Compared to human and equine casein, ruminant casein (except for some goat milks) is relatively abundant with αs1-casein being the predominant factor in the development of or sensitization to milk allergy [8,10,165]. Thermal processing may destroy epitope structures, but can also unmask them or create new ones. For that reason heating can increase or decrease allergenic potential, depending upon the protein (or component) involved and on the individual patient [1]. Casein is fractionated into α-, β-, and κ-casein. Whey proteins include: α- lactalbumin (α-la), β-lactoglobulin (β-lg), bovine serum albumin (BSA) and immunoglobulin (Igs). Most studies revealed, that casein and β-lg are the main allergens in cow milk [166,167]. Jarvinen et al. [168] found, that five IgE-binding epitopes (2 on α-s1-casein, 1 on α- s2-casein, and 2 on κ-casein) were recognized in patients with persistent allergy. The IgE antibodies were against at least one of three epitopes. Amino acid (AA) 123–132 on α-s1-casein; AA 171–180 on α-s2-casein, and AA 155–164 on κ-casein. Allergic reactions to BSA, IgG heavy chain and α-la were also recorded [169,170]. The structure of sequential epitopes recognized by IgE antibodies to α-la and β-lg was found in CMA (cow milk allerdy) patients. Four IgE-binding regions were identified on la and seven IgE-binding epitopes were detected on β-lg [164,169]. Allergic responses to lactoferrin and some cow milk enzymes have been detected in some patients with CMA [171]. The major whey proteins of bovine milk are β-lg with 55% of total whey proteins, α-la with 20%, and BSA with 7% [164]. These proteins vary in their types and ratios between goat, sheep, cow, camel, human, buffalo, mare and donkey

 doi:10.20944/preprints202210.0097.v1

milks. Human milk is free of β-lg [172], one of the major allergens in cow milk, similar to camel milk, which also has no β-lg [164].

The use of goat's milk (GM) for feeding healthy babies or as a possible substitute to CM for allergic people is still debatable [127,173]. The higher content of caprine milk in essential fatty acids makes it easily digested compared to bovine milk. Moreover, in diets administered children with atopic dermatitis, it seems to be less allergenic than CM making it a possible dietary supplement in individuals with inflammatory and allergic conditions. However, studies have proved that many children allergic to CM are also sensitized to proteins of GM, as a result of the close phylogenetic relations between these animals and the high sequence identity of their homologue proteins [174]. Recently, various research groups have reported a comparison of the milk proteome profiles of some animal species, including goat, for identifying sources of hypoallergenic alternatives to bovine milk [175,176]. Moreover, by monitoring the characteristic peaks of the most abundant proteins, MALDI-TOF MS based methods have been recently developed to detect fraudulent adulterations or unintended contaminations of other milks to DM and GM and therefore to assess the genuineness of these milks [177,178].

Proteome of GM presents several components which are homologues to bovine milk glycoproteins relatively resistant against proteolysis in the gastrointestinal tract, and playing an important physiological role in the defense /immunity mechanisms. Among these proteins, lactoperoxidase and Milk fat globule-EGF factor 8/lactadherin. Lactoperoxidase contribute to the defense against both gram-positive and gram-negative pathogenic bacteria, and it is used in dairy industry in order to preserve microbial quality. Milk fat globule-EGF factor 8/lactadherin prevents symptomatic rotavirus infection in breastfed infants, and more in general, it seems positively interact with damaged intestinal epithelium. Thus, lactadherin could have a potential role in the prevention and treatment of intestinal injury in infants [29,179]. The group of proteins only found in GM also includes the haemoglobin subunit beta from ovis (Acc. N. P02075), which corresponds to the homologue bovine, a blood-derived protein identified in red meat as partly muscle-specific and heat-resistant allergen (Bos d HG, Acc. N. P02070). Primary structure comparison of these two proteins reveals that they share 93% of identity and 95% of similarity, showing only ten amino acid point mutations, making the protein a potential minor allergen [29].

Thermal treatment

Heat treatment is included in most dairy industries to obtain bacteriologically safe final products and to extend their shelf life. A number of structural modifications have been recognized in the milk protein component depending on time, temperature and rate of heating. Singh [180] showed that a range of large heterogeneous protein aggregates of milk proteins occurred in heat-treated milk. The heat-induced milk protein association occurring under different heating conditions has been extensively investigated [181,182].

Heat treatment of milk mainly affects the milk fat globule membrane (MFGM) and a number of heat sensitive MFGM protein components, changing the agglomeration and creaming of fat globules [183]. Some of the heat-induced changes of the MFGM include the association of whey proteins and casein with the MFGM through sulphydryl–disulphide interchange reactions, the release of sulphydryl compounds, most notably H_2S, and the removal of phospholipids at elevated temperature [1].

Both caseins and whey proteins are engaged in protein aggregates found in heat treated milk and that the formation of intermolecular disulfide bonds is mostly responsible for heat-induced protein association in milk [184]. The thermal protein denaturation has been acknowledged as the primary step of the reactions leading to the aggregation of disulfide-linked milk proteins. Thiol groups of cysteine residues, appearing in unfolded proteins, can initiate thiol-disulfide exchange reactions within hydrophobically linked protein aggregates. Self-aggregation of heat-denatured β-lg in water [185], heat-induced association of whey proteins and/or their aggregates with caseins [186] have been explained according to this mechanism [182].

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The digestibility of individual milk proteins from different species differs as well. For example, horse β-lg is more easy to digest than goat β-lg [187], and goat and sheep β-lg are more easily digestible than bovine β-lg [2,188]. Whereas α-lactalbumin of all species appears to be relatively hard to digest, the other whey proteins, including lactoferrin and serum albumin, seem to be easily digestible, in human and horse milk as much as in bovine and goat milk [187]. Heating (95°C/1min) appears to have only a minor effect on the gastrointestinal degradation of caseins, but seems to improve the digestibility of whey proteins [187,189]. Digestibility of human, horse, bovine and goat α-lactalbumin for example, is reported to improve upon heating (12 to 20% at 95°C/1min) [187]. Heating (pasteurization, UHT) mainly modifies the functional properties of milk proteins (e.g. emulsifying and water binding properties, solubility), but has little effect on their digestibility and nutritional properties [190]. Roos et al. [191] demonstrated that about 19% of ingested bovine IgG and IgM was found to retain immunological activity in the ileum of healthy human adults. No IgA was detected in the ileum. Besides, the activity or content of most antimicrobial systems or components varies strongly in milk and is mainly high in colostrum with a rapid decline during further lactation [2,10,192,193]. As such, the effect of heating on antimicrobial systems of milk seems to be of little relevance in this context. On the other hand, lysozyme is reported to increase strongly after the second month of lactation (in human milk), and, being very resistant to acid and proteolysis (in horse milk), to reach presumably the gut relatively intact [1].

Milk protein solutions with high thermal stability are characterized by low viscosity, low turbidity and high solubility after heating. These conditions are influenced by physicochemical properties of the particles, such as surface hydrophobicity, aggregate size, shape and charge [194-196]. As a result, the heat stability of proteins varies greatly with the pH at heating, the ionic strength of the dispersion, and the heat load applied [197]. Combined aggregates of whey proteins and caseins show higher heat stability than whey protein aggregates. It is generally accepted that this is due to a chaperone-like activity of the caseins. Chaperone activities can stabilize proteins from unfolding, aggregation and precipitation [198]. In dairy science and technology, aggregation is probably the more important phenomenon, as uncontrolled aggregation can lead to destabilisation. αs-, β- and κ-casein have been shown to exhibit chaperone activity against aggregation [199]. The presence of caseins provided stabilization of whey protein aggregates during heating. In particular, κ-casein exhibited a chaperone-like activity at a whey protein to κ- casein ratio of 1:0.7, for both heated and unheated mixtures of whey proteins and κ-casein. The presence of αs- and β- casein in solution contributed to an enhanced heat stability of the whey proteins.

Protein denaturation can also reduce in vitro protein digestibility [200], and reduce the absorption of minerals including calcium, iron and zinc, as these are transported and made available for uptake by some of the milk proteins [119,201].

Recently, heat-induced hydrolysis of either whey- or casein-based products have been studied by proteomic methods. McGrath and collaborators [202] reported the hydrolysis of sodium caseinate during heating for up to 120 min at pH 7 and 130 °C. Peptide bonds containing Pro, Ser, Asn and/or Asp were preferentially hydrolysed. as1-CN was the most susceptible to the heat-induced hydrolysis and k-CN was the most resistant. Indications of acid-induced hydrolysis of whey proteins, in combination with deamidation, have also been reported recently (Le et al., 2016). Here, the heat-induced deamidation leading to an increased amount of Asp and Glu residues produced from Asn and Gln, respectively, formed the basis of acid-induced hydrolysis of a- La, b-Lg and CMP [15].

As to the thermal stability of milk proteins, differences between species are mainly due to differences in amino acid sequence (and number of S-S bridges or sulphydryl groups) and in milk environment (e.g. slightly different pH, fat content). For example, lactoferrin and serum albumin have a similar thermostability in bovine and horse milk, whereas β-lg and α-lactalbumin have a higher thermostability in horse compared to bovine milk. The high thermal stability of equine β-lg may be related to its monomeric form

 doi:10.20944/preprints202210.0097.v1

and the lack of a free SH group [2,203]. Whey proteins of bovine milk are less resistant to heat denaturation compared to those of buffalo milk [204], which in turn are less heat resistant than camel whey proteins [205,206]. Even though camel whey proteins have a higher heat stability than bovine whey proteins at temperatures between 63 and 90°C, bovine milk coagulates much slower at higher temperatures. This could be related to the absence or very low levels of β-lg and κ- casein in camel milk [207] as milk is more resistant to heat when it is characterized by a molar β-lg to κ-casein ratio close to 1 [165].

Adulterations

Genetic polymorphism and posttranslational modifications of the major milk proteins can affect the physicochemical and biological properties of milk [208]. Approximately 80% of cow milk proteins are made of caseins (αS1-, αS2-, β-, and κ-CN). Caseins are encoded by 4 genes (CSN1S1, CSN1S2, CSN2, and CSN3) that are all located in a 250-kb spanning region of chromosome 6 and show high mutation rates. As a consequence, allele and haplotype frequencies can vary significantly between and within species and breeds, and detailed analyses have revealed a geographically associated haplotype distribution that can shed light on the evolution of the bovine casein locus [209]. To date, more than a dozen genetic variants of β-CN have been described; A1 and A2 are the 2 most common β-CN variants [208,210]. The A1 variant evolved from A2 through a mutation at position 67, and β-CN variants are often grouped into the A1 or A2 type based on the amino acid present at this position (His67 in the A1 type and Pro67 in the A2 type). The histidine residue in A1-type β-CN allows enzymatic release of the preceding 7 amino acid residues in the gastrointestinal tract to generate the β-casomorphin-7 (BCM-7) peptide [211]. Release of this peptide is either prevented or greatly reduced from A2-type β-CN [211-213]. Although BCM-7 was initially associated with several adverse effects, a review by the European Food Safety Authority [214] concluded that "a cause-effect relationship between the oral intake of BCM7 or related peptides and etiology or course of any suggested non-communicable diseases cannot be established." Recent studies have reported that consumption of A2 milk may be associated with attenuation of gastrointestinal symptoms of milk intolerance in self-reported lactose intolerant individuals [215-217]. Cows can be genetically selected to be homozygous for β-CN A2 (A2A2 genotype) and thereby produce milk that only contains β-CN of the A2 type (A2 milk), and dairy products manufactured from A2 milk have been on the market for many years. Intact protein analyses by reverse-phase (RP)-HPLC or capillary electrophoresis (CE) coupled to UV detection can effectively separate most β-CN variants in raw milk samples [218,219]. Coupling RP-HPLC with MS could in principle overcome this limitation provided the different proteoforms can be sufficiently resolved by the mass spectrometer. A study by Givens et al. [220] applied HPLC-MS to the analysis of pasteurized milks but did not study UHT or powder milks that can often contain more lactosylated proteoforms [221]. A1:A2 β-CN ratios were analyzed in raw milk and powder infant formulas by multiple reaction monitoring after digestion of casein precipitates by thermolysin [222]. Although A2 raw milk contained exclusively A2-type β-CN, β-CN A1 levels were surprisingly elevated in an infant formula prototype manufactured using A2 milk. The authors attributed this observation to the presence of other milk-derived ingredients used in the formulation. This observation was also reported in another study [223]. Indeed, proteolysis of β-CN by plasmin leads to the generation of γ-CN and protease peptones (PP8 fast, PP8 slow, and PP-5). In contrast to γ-CN, protease peptones do not coagulate with caseins during acidification or rennet treatment and remain in the whey fraction. PP8 slow and PP-5 correspond to sequences 29 to 105/7 and 1 to 105/7, respectively, and both species include position 67. Thus, whey products derived from milk containing β-CN A1 contain protease peptones of the A1 type that will be detected by peptide-directed analyses after digestion with proteolytic enzymes such as trypsin, thermolysin, and GluC. Consequently, peptide based approaches are particularly problematic for the true detection of β-CN variants in final products. The UPLC-HRMS intact protein method presented by Fuerer et al.[217] is an effective

 doi:10.20944/preprints202210.0097.v1

method for the identification and quantification of proteins in dairy samples such as raw milk, SMP, whey powder, final products, and samples from other species such as buffalo or human. It allows a straightforward and unambiguous detection of the major milk proteins (below 30 kDa) and their associated proteoforms, with low-abundance genetic variants being detectable around 2%. The increased demand of goat milk consumption as a substitute milk for bovine allergen or in cheese-making progress pushed food authorities to invent testing models to check the quality of goat products. The common method used by EFSA is PCR detecting impurities [224]. For that reason, Guarino et al [225] in a study performed in goat cheese found that the casein peptides derived from either sheep or goat milk with LC/MS-MS technique can be used as markers of goat cheese purity.

Among differentially expressed proteins in Holstein -Friesian (HF) cows across seasons (W: winter, S: summer), HFW showed higher expression of α-lactalbumin, e-cadherin and follistatin related protein (FRP) compared to HFS. Higher abundance of α-lactalbumin in a study by Maity et al [226] might be suggestive of increase in lactose synthesis required for higher energy in winter is at odds with previous reported results [227]. Similarly, HFS showed higher abundance of cathepsin L, plasmin and acute phase proteins (APP) like haptoglobin and alpha-1-acid glycoprotein (α1AG); as a coping mechanism towards heat stress [228]. In the same study, the comparison between Murrah buffalo (MuW) and MuS identified a number of differentially expressed proteins. For example, FRP, acyl CoA binding protein (AcoABP), gelsolin, perilipin-2 (PLP2), osteopontin and conglutinin were overexpressed in winter. Consistent with previous reports, AcoABP and PLP2 was up-regulated and likely linked to lipid metabolism explaining higher fat content in Mu in winter [229]. The presence of PLP2, HSP70 and thrombospondin in both bovine and buffalo milk has been linked to provide protection to cellular proteins from oxidative stress and animals from infection [230,231]. Interestingly, higher concentration of HSP70 also contributes to the stability of buffalo either bovine milk [232] during pasteurization or sterilization suggesting its easier handling during processing in dairy industries and based on the abundance of certain proteins a discrimination between winter or summer milk of these species can be easily made.

Conclusions

In overall, milk is a globally consumed food and its quality need to be ensured. Proteomics methods are widely used in order to elucidate the hidden properties of milk proteins in all dairy species. Peptides derived from milk might be included in therapeutic approaches in patients with hypertension, gastrointestinal diseases or even cancer. Also, caprine milk is a substitute of bovine in infants with allergies, as it contains proteins easily tolerated by infant's intestine. Furthermore, proteomics in food industry revealed which proteins are thermal stable and remain unaltered providing benefits for dairy production. The most important aspect is that applied proteomics ensure the quality and purity of milk and dairy products, especially the PDO (protective designation of origin) ones.

Author Contributions: Writing – original draft, Efterpi Bouroutzika; Writing – review & editing, Efterpi Bouroutzika, Stavros Proikakis, Ekaterini Theodosiadou, Angeliki I. Katsafadou, Irene Valasi and George Th. Tsangaris.

Funding: This work supported by the project "FoodOmicsGR-RI Comprehensive Characterization of Foods" (MIS 5029057) which is implemented under the Action "Reinforcement of the Research and Innovation Infrastructure", funded by the Operational Programme Competitiveness, Entre-preneurship and Innovation (NSRF 2014–2020) and co-financed by Greece and the European Union (European Regional Development Fund).

Conflicts of Interest: The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this review.

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